Post-mortem Interval estimate based on dental pulp: A histomorphology approach

Ilenia Bianchi [1], Ana Rodrigues [2], Rui Santos [3], Diana Augusto [2], Martina Focardi [1], João Aquino [4], Isabel Fonseca [5], Cristiana Palmela Pereira [6]

[1] Laboratory of Personal Identification and Forensic Morphology, Department of Health Sciences, University of Florence, Florence, Italy. [2] Research Group in Forensic Dental Sciences FORENSEMED from the Research Unit UICOB, Faculty of Dental Medicine, University of Lisbon, Portugal. [3] School of Technology and Management, Polytechnic Institute of Leiria, Portugal; Centre of Statistic and its Application CEAUL from University of Lisbon, Portugal. [4] Faculty of Dental Medicine, University of Lisbon, Portugal. [5] Faculty of Medicine, Institute of Pathological Anatomy, University of Lisbon, Portugal. [6] FORENSEMED/UICOB from the Faculty of Dental Medicine, University of Lisbon; Centre of Statistic and its Application CEAUL from University of Lisbon; Faculty of Medicine University of Lisbon, Portugal

Corresponding author:
ilenia.bianchi@unifi.it

The authors declare that they have no conflict of interest.

KEYWORDS

Post-mortem interval;
Histological changes;
Dental pulp;
Haematoxylin and Eosin stain;
High Resolution Microscopy

J Forensic Odontostomatol
2024. Aug;(42): 2-60:75
ISSN :2219-6749
DOI: doi.org/10.5281/zenodo.13371720

ABSTRACT

Estimating the post-mortem interval (PMI) of human remains based on the histomorphology of dental pulp parameters is promising, but available evidence is scarce and sometimes contradictory without a scientific model.

The aim of the study is to characterise the histomorphological changes of dental pulp associated with the decomposition of human remains by a qualitative and quantitative approach. The main aim is to establish a correlation based on post-mortem (PM) dental pulp histomorphology and the PMI, and whether pulp degradation could be an available medico-legal tool for PMI estimation beyond the first week after death (late PMI).

The eligible sample consisted of 27 sound teeth from 16 healthy patients aged 16 to 72 years due to orthodontic or oral surgery treatment, to create PMI's simulating the death of the subject as the time elapsed from tooth avulsion. Data collected from patients (sex, date of birth, tooth position, date and hour of the avulsion, date and hour of pulp extraction) were anonymised in accordance with the requirements of Faculty of Dental Medicine of the University of Lisbon.

The sample was divided into 9 groups of 3 teeth according to different PMI sets from T0 (baseline) up to 2 weeks (T0, 7, 12, 24, 36, 48, and 72 hours, 1 and 2 weeks). All the dental samples were stored at room temperature up to the time of pulp extraction and then prepared with haematoxylin and eosin stain. High-resolution microscopy was performed to obtain histological images. An operator performed the qualitative evaluation of blood vessels, collagen fibres, and the extra-cellular matrix (ECM) in PM pulps and measured the variation in cells/nuclei density by counting 6 different ROIs (Regions of Interest) for each pulp manually and automatically (quantitative analysis).

Qualitative results showed that the degeneration of dental pulp appears 7 hours after death but histological changes in vessels, fibres, and ECM in PM dental pulp are characterised by high variability, consequently it is not possible to generalise the results for early PMIs. Quantitative measurements proved that cell count cannot be standardised due to the presence of superimposed layers of cells and nuclei fragmentation. Odontoblasts did not demonstrate evidence of cellular or nuclear lysis up to 14 PM suggesting their applicability in late PMIs.

Future research will focus on late PMIs and different techniques of tooth preparation.

INTRODUCTION

A reliable estimation of the post-mortem interval (PMI) is an important step in forensic investigations since criminal justice requires the charge to be proven beyond reasonable doubt. The most common tool for early PMI estimation (from 3 to 72 hours after death) is the algorithmic combination of different parameters (in particular, body-environmental temperatures) through the so-called "compound method". One crucial limitation of this technique is that it cannot be applied to longer PMIs, with measurement errors generally in months, since they are based on decomposition stages (fresh, early and advanced decomposition, skeletonisation, and extreme decomposition) [1]. In particular, it has been observed that macroscopic/morphological modifications of skeleton, teeth, or soft tissues provide less reliable PMI estimates due to highly variable individual influences, even under the same environmental conditions. Nevertheless, they are useful for other identification investigations, such as age estimation or sex classification [2-9].

Currently, alternative methods are focused on post-mortem (PM) biochemical or histological changes [10-23]. One of the most stable matrices for estimating the time elapsed since death from early to late PMIs appears to be dental pulp, which is protected from damaging factors (such as heat, bacteria, UV light, and moisture) by the hard tissues of the tooth [24-26]. Despite having great resistance, teeth undergo histomorphological changes due to physical-chemical factors related to the absence of physiological regulation of metabolic processes, ischemia, putrefaction, and bacterial and fungal proliferation [27-36].

It has been observed that as time elapses after death, odontoblasts cluster and detach from dentine, collagen fibres and nuclei of odontoblasts, fibroblasts, endothelial and immunity cells, and stromal cells decrease in density, and the extra-cellular matrix (ECM) degenerates into a vacuolated and contracted tissue [30-36]. Nevertheless, the rate of degeneration and its correlation with the PMI is not clearly defined. Some authors describe changes that occur in the first hours after death up to an almost complete disappearance of fibres and cells in a week PM [30, 32-33]; other studies report the persistence of collagen fibres and cells up to 6 months post-mortem and complete destruction of the ECM 2 years after death [34-36].

Previous studies on human teeth that verify dental pulp's histomorphological changes correlated with the time elapsed since death provide a rationale for developing a tool for estimating PMI based on teeth, however, it is challenging with limited results, and a scientific model is still missing. Therefore, the aim of the study is to verify which qualitative and quantitative dental pulp changes are significant for the correlation with PMI and whether pulp degradation could be a reliable forensic technique beyond the first week after death (late PMIs). Pilot research was developed to standardise the study method for both qualitative and quantitative PM dental pulp changes.

OBJECTIVES OF THE PILOT STUDY

Qualitative analysis

Qualitative analysis consisted of the identification and description of morphological structures of PM pulp to answer experimental hypotheses, noted in previous literature [30-36]:

1. The dilatation of vessels observed in the dental pulp as a dental element in the medico-legal estimation of the PMI:
   H0: The dilatation of vessels is not significantly different over time after death;
   H1: The dilatation of vessels is significantly different over time after death.
2. The rate of homogenisation of collagen fibres observed in the dental pulp is as follows:
   H0: The homogenisation of collagen fibres is not significantly different over time after death;
   H1: The homogenisation of collagen fibres is significantly different over time after death.
3. The vacuolation of the ECM of dental pulp is as follows:
   H0: The tissue vacuolation is not significantly different over time after death;
   H1: The tissue vacuolation is significantly different over time after death.

Quantitative analysis

Quantitative analysis consisted of the measurement of cell density as nuclei count/ROI area [30-36]. More specifically cell density was measured by the variation in the density of fibroblasts, immune and endothelial cells, and odontoblasts (odontoblast layer), as follows:

H0: The cell density of the dental pulp is not significantly different over time after death;
H1: The cell density of the dental pulp is significantly different over time after death.

MATERIALS AND METHODS

Sample collection and storage

Sound teeth from different people who underwent dental surgery for clinical reasons (e.g. orthodontics, impacted tooth, etc) were collected from two medical centres: the Faculty of Dental Medicine and Faculty of Medicine of the University of Lisbon (ULisboa). The following inclusion/exclusion criteria were considered:
Inclusion criteria: permanent, complete, and sound tooth (both single- and multi-rooted).
Exclusion criteria: hard tissue pathology (e.g. caries), restorative treatment (e.g. filling, endodontic, prosthetic), periodontal pathology, severe wear, incomplete root formation, deciduous teeth.
A total of 27 sound teeth were collected from 16 healthy patients aged 16 to 72 years. Information about the sample and the time of tooth/pulp extraction was recorded in the *Dataset* figured in Table 1.

Table 1. Dataset of the sample and time of tooth/pulp extraction

Sample					Time of death		Pulp fixed in formalin		
ID Code	Tooth	Sex	Date of birth	Age (year)	Date of tooth extraction	Hour of tooth extraction	Date of pulp extraction	Hour of pulp extraction	PMI
1	35	M	22.06.1976	46	19.04.2023	12:00	20.04.2023	11:50	24 h
2	24	M	08.02.2000	23	20.04.2023	15:50	21.04.2023	16:12	24 h
3	34	M	08.02.2000	23	20.04.2023	16:05	21.04.2023	16:44	24 h
4	18	F	07.06.1998	24	26.04.2023	15:30	28.04.2023	16:05	48 h
5	25	M	29.04.2007	16	04.05.2023	11:45	04.05.2023	18:43	7 h
6	35	M	29.04.2007	16	04.05.2023	12:09	04.05.2023	19:09	7 h
7	18	F	08.02.2003	20	12.05.2023	11:30	12.05.2023	11:50	0 h
8	48	F	08.02.2023	20	12.05.2023	11.45	12.05.2023	12.05	0 h
9	45	M	29.04.2007	16	18.05.2023	12:10	25.05.2023	12:13	7 d
10	17	M	26.10.2001	21	24.05.2023	15:30	26.05.2023	16:10	48 h
11	25	F	09.04.2002	21	31.05.2023	9:20	03.06.2023	9:18	72 h
12	47	M	02.09.1962	61	02.06.2023	10:49	05.06.2023	11:00	72 h
13	15	M	29.04.2007	16	24.05.2023	11:16	07.06.2023	11:00	14 d
14	38	F	13.10.1952	72	31.05.2023	16:00	07.06.2023	16:15	72 h
15	35	M	25.02.2004	19	31.05.2023	9:38	14.06.2023	09:42	14 d
16	45	F	24.11.2004	18	21.06.2023	9:35	28.06.2023	9:40	7 d
17	15	F	24.11.2004	18	21.06.2023	10:03	28.06.2023	10:05	7 d
18	25	M	30.06.2001	21	21.06.2023	11:41	05.07.2023	11:41	14 d
19	18	M	20.03.2005	18	06.07.2023	09:32	07.07.2023	09:35	36 h
20	45	F	07.07.1998	25	12.07.2023	10:24	12.07.2023	17:25	7 h
21	15	F	07.07.1998	25	12.07.2023	9:47	12.07.2023	21:45	12 h
22	35	F	07.07.1998	25	13.07.2023	9:44	13.07.2023	21:44	12 h
23	14	F	26.07.2001	21	12.07.2023	11:45	14.07.2023	11:45	48 h
24	25	F	07.07.1998	25	13.07.2023	10:04	14.07.2023	22:04	36 h
25	38	M	06.04.2005	18	26.07.2023	9:45	26.07.2023	10:45	0 h
26	48	M	06.04.2005	18	26.07.2023	9:45	26.07.2023	21:45	12 h
27	18	M	06.04.2005	18	26.07.2023	9:45	27.07.2023	21:45	36 h

The period of sample storage included spring and summer seasons with an average recorded temperature of 22°C (range of 20-24°C) for the months of April and May, and of 27.5°C (range of 26-29°C) for the months of June and July.
The sample size considered 3 teeth in each PMI set, for a total of 9 PMI sets from T0 (baseline) up to 2 weeks.
All the data collected from the patients was processed in compliance with European Union law (General Data Protection Regulation - GDPR) and in accordance with the requirements of the Faculty of Dental Medicine of the University of Lisbon: a progressive numerical code was associated with each anonymous dental sample.
Tooth avulsion was assumed as the time of death of the patient since the extraction interrupts the blood perfusion of the dental pulp, and the PMI was considered as the time elapsed between tooth avulsion and the pulp extraction and fixation.
Teeth from clinical cases were selected, rather than those from forensic cases, to achieve known PMIs, as opposed to an estimated time of death.
Immediately after avulsion, teeth were placed in sterilisation pouches, labelled with sample data: the registered information for each patient included sex, date of birth, tooth position, date and hour of avulsion, and date and hour of pulp extraction (PMI). All samples were stored at room temperature recorded by a thermometer up to the established PMI to simulate real corpse conservation during time after death.

PMIs set and Pulp extraction
The sample was divided into 9 groups of 3 teeth according to the established PMI:
- Group 1: PMI T0 – pulp immediately after tooth extraction
- Group 2: PMI 7 hours – pulp 7 hours after tooth extraction - within which the intraocular pressure decreases drastically and stabilises after death [1] and histological examination of the skin shows no morphological changes [3]
- Group 3: PMI 12 hours - pulp 12 hours after tooth extraction - stability phase of rigor mortis [3-4]
- Group 4: PMI 24 hours - pulp 24 hours after tooth extraction - stability phase of rigor mortis [3-4]
- Group 5: PMI 36 hours - pulp 36 hours after tooth extraction - from the first day PM until the third day PM, the investigations are performed every 12 hours to study the gradual degradation of the pulp
- Group 6: PMI 48 hours - pulp 48 hours after tooth extraction
- Group 7: PMI 72 hours - pulp 72 hours after tooth extraction
- Group 8: PMI 7 days - pulp 7 days after tooth extraction - to verify the availability of PM dental factors for intermediate PMIs up to 1 and 2 weeks after death (3 teeth)
- Group 9: PMI 14 days - pulp 14 days after tooth extraction.

At the time of pulp extraction, each tooth was rinsed with a 70% alcohol solution and the aid of sterile gauze.
The tooth is then positioned with the crown securely stabilised inside a clamp and a horizontal cut is made to separate the crown from the root with a flame diamond bur under an abundant jet of water at the height of the furcation for multi-rooted teeth and about 2 mm below the cementum-enamel junction for single-rooted teeth.
Once the crown is separated from the root/roots, the floor of the pulp chamber is worn down until the pulp is completely exposed, with the diamond bur under abundant cold water. The coronal pulp was carefully separated from the chamber walls with a thin probe and then delicately pulled out from the coronal chamber with tweezers.
Only the coronal pulp was selected for this study. Once extracted, the entire chamber pulp fragment was immersed in the fixative liquid for standard histology (10% neutral buffered formalin - NBF).

Histological processing and stain of soft tissues [Haematoxilin/Eosin stain]
The histological preparation procedure for dental soft tissue followed the protocol applied by the Laboratory of Histomorphology from the Faculty of Dental Medicine of the University of Lisbon and consisted of the following phases: dehydration, clearance, fixation, impregnation, inclusion, microtomy, expansion, adhesion, staining, and assembly.
- The dehydration phase: sequential application of ethanol application to 70%, to 96%, and to 100%.

- The dehydration solution was replaced by a solvent for a means of inclusion (e.g. Xylol, Chloroform, Toluene, Benzene) since the specimens became transparent (clearance).
- The samples were included in paraffin and the inclusion was oriented according to the structure of pulp tissues (lengthwise), thus the major axis of the sample is parallel to the cut area of the paraffin block.
- Once solidified, cuts of 2/3 microns thick were made. Only sound sections were considered.
- The sections were then placed, first in cold water and then in hot water. This phase needed a black background to ease the visibility of the pulp sections and their positioning on the slides.
- The adhesion of the pulp sections to the slides took place in an incubator set at 58-59 °C to liquefy the paraffin and complete the adhesion.
- For histological staining, the procedure followed a de-paraffinisation/de-waxing with xylene, hydration with ethanol from 100%, to 96%, and to 70%, then a stain with Haematoxilina de Harris, a differentiation with hydrochloric acid at 1%, and a wash with water. Subsequently, the slides were coloured with eosin, dehydrated with ethanol at 96% and then at 100%. After a clarification with xylene, the assembly was performed with synthetic resin entellan which completes the adhesion of the section to the slide.

High Resolution Microscopy

Each slide was scanned and acquired with *Aperio GT 450 DX Digital Slide Scanner* (Leica Biosystems) at the Faculty of Medicine, Institute of Pathological Anatomy of the University of Lisbon.

High resolution images of the entire pulp section were used by the operator to:

- describe qualitative characteristic of PM pulp
- select two different kinds of ROIs (regions of interest), at the same magnification (40x) but with a larger area [249480 μm^2 area] and smaller [3180 μm^2 area], for each section's nuclei count

To find the most reliable and repeatable method to calculate cell density, 6 different ROIs at magnification of 40X for each slide were selected: 3 larger [249480 μm^2 area] and 3 smaller [3180 μm^2 area] including 1 region of the odontoblast layer and 2 regions of cell rich layer, fibres, vessels, and ECM. Larger and smaller ROIs were considered separately.

The nuclei count was conducted both manually by the operator and automatically using *Fiji ImageJ software* (free open source, accessible and downloadable from https://imagej.net/software/fiji/downloads).

To reduce the risk of measurement error within the same slide, the average nuclei count was calculated on 3 different ROIs (both on smaller and larger areas of selection). To reduce the risk of error within the same PMI set, cell density was obtained as the mean of the 3 teeth (PMI groups).

Statistical Analysis

Cohen's Kappa ($\varkappa$) statistic was applied to calculate the intra-rater and inter-rater reliability of the quantitative analyses. 3 PMI groups (T0, 7 days, and 14 days) were selected for the intra-rater agreement, conducted by the same operator 15 days after the first measure, and for the inter-rater agreement, conducted blind by another operator.

RESULTS

Qualitative results

A summary of the most significant histological features at PMI T0 (baseline), 7h, 12h, 24 h, 36h, 48h, 72h, 7 days, 14 days is recorded in Table 2.

Quantitative results

According to Cohen's Kappa ($\varkappa$) statistic, the intra-rater agreement values for both the smaller and larger ROIs were almost 1.

The inter-rater agreement values for the larger ROIs count and density measurement were 0.892 and 0.758 respectively, for the smaller ROIs count and density measurement were 0.931 and 0.957, respectively.

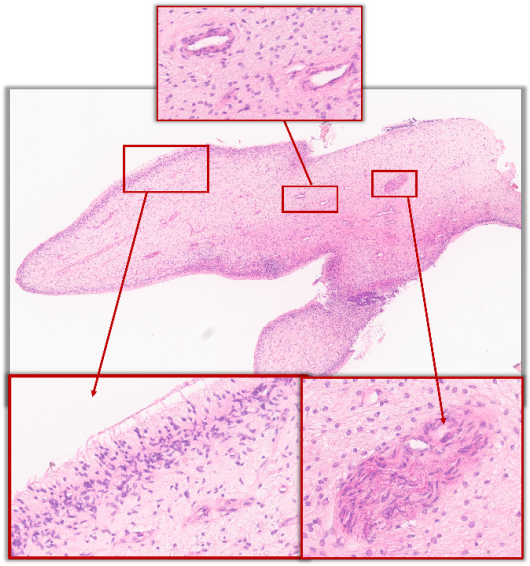
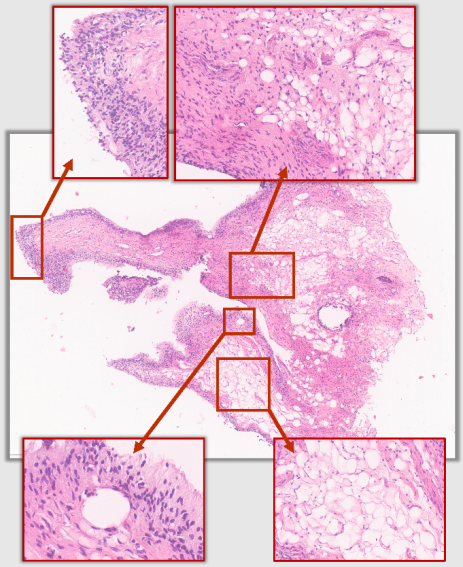
Manual cell counts and density measurements for each PMI calculated on the larger ROIs are recorded in Figures 1 and 2 respectively, on the smaller ROIs in Figures 3 and 4 respectively.

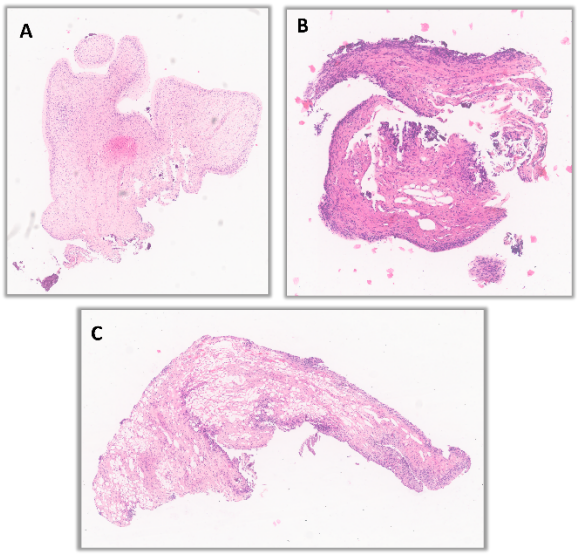
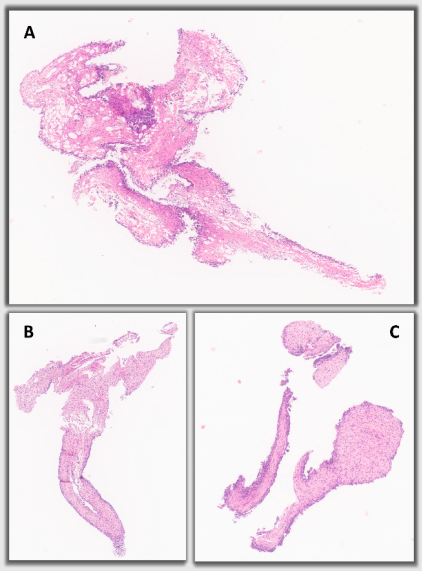
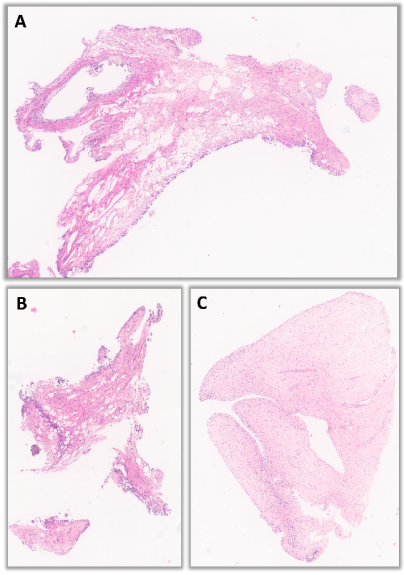
Automatic cell counts and density measurements calculated on the larger ROIs are shown in Figures 5 and 6 respectively, on the smaller ROIs in Figures 7 and 8 respectively.

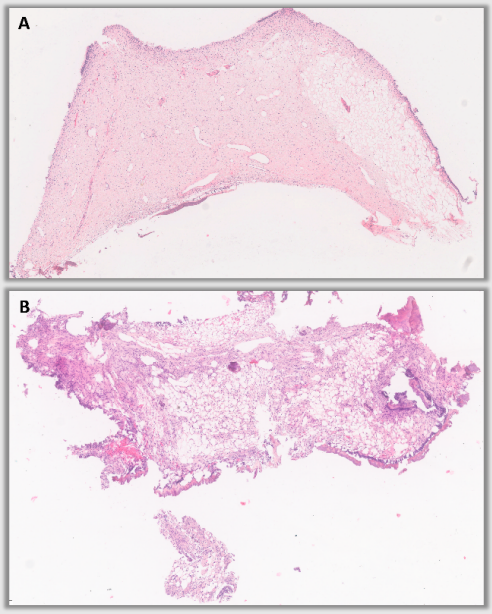
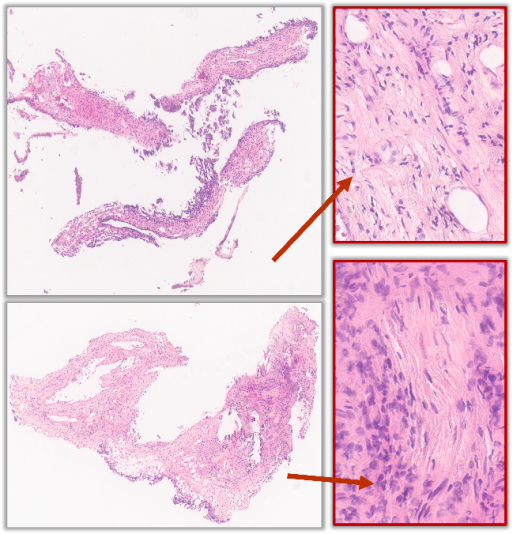
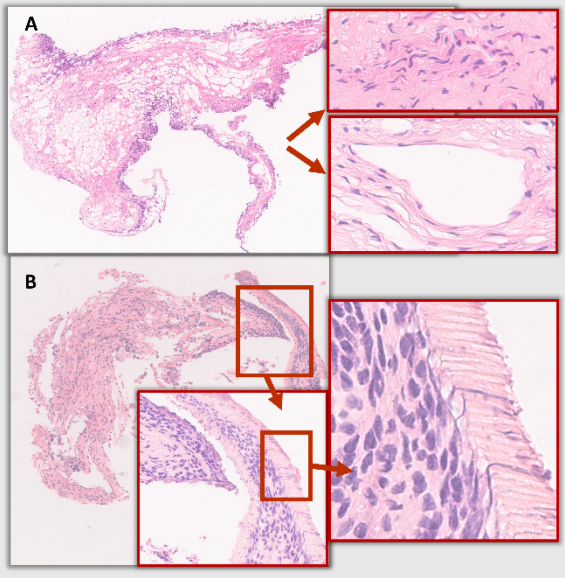
According to the different methods of count, nuclei density calculated manually considered as unit of measurement the Square Micrometer (μm^2) and the area included was the same for each

ROI, whilst the method calculated by automatic software considered Pixels and the automatic setting (the standard proposed by the software as the best fitting) considered different areas for each ROI.

Table 2. Summary of the most significant histological characteristics of dental pulp at PMI To (baseline), 7h, 12h, 24 h, 36h, 48h, 72h, 7 days, 14 days

PMI	Stain Haematoxilin/Eosin	Histological features
To (baseline)		• Homogeneous distribution of extracellular matrix without vacuolisation; • Well represented odontoblasts with homogeneous distribution and stratification of nuclei within the odontoblast layer; • Well represented different cell types with circular, ovoid, and elongated shaped nuclei; • Well conserved endothelial cells of blood vessels; • Well conserved nerve cells.
7h		• Extracellular matrix disaggregated with vacuolisation areas; • Well conserved circular, ovoid, and elongated shaped nuclei, endothelial cells of blood vessels, and nerves distributed among the degenerative areas; • Well represented odontoblasts with homogeneous distribution and stratification of nuclei within the odontoblast layer.

12h		Different stages of extracellular matrix degeneration: • Panel A: homogeneous distribution of extracellular matrix without vacuolisation; • Panel B: extracellular matrix with localised disaggregated and vacuolised areas; • Panel C: extracellular matrix with massive disaggregated and vacuolised areas; • All three samples: well conserved circular, ovoid, and elongated shaped nuclei, endothelial cells of blood vessels, and nerves, and well represented odontoblasts with homogeneous distribution and stratification of nuclei within the odontoblast layer.
24h		Different stages of extracellular matrix degeneration: • Panel A: extracellular matrix with massive disaggregated and vacuolised areas; • Panel B-C: homogeneous distribution of extracellular matrix without vacuolisation; • All three samples: well conserved circular, ovoid, and elongated shaped nuclei, endothelial cells of blood vessels, and nerves, and well represented odontoblasts with homogeneous distribution and stratification of nuclei within the odontoblast layer.
36h		Different stages of extracellular matrix degeneration: • Panel A-B: extracellular matrix with massive disaggregated and vacuolised areas; • Panel C: homogeneous distribution of extracellular matrix without vacuolisation; • All three samples: well conserved circular, ovoid, and elongated shaped nuclei, endothelial cells of blood vessels, and nerves, and well represented odontoblasts with homogeneous distribution and stratification of nuclei within the odontoblast layer.

48h		Different stages of extracellular matrix degeneration but degenerative tissue is well represented in samples: • Panel A: extracellular matrix with localised disaggregated and vacuolised areas, conserved circular, ovoid, and elongated shaped nuclei, blood vessels, and nerves, and represented odontoblasts with homogeneous distribution and stratification of nuclei within the odontoblast layer; • Panel B: extracellular matrix with massive disaggregated and vacuolised areas. All structures (nuclei, odontoblast layer, vessels, and nerves) are visible.
72h		• Extracellular matrix with several disaggregated and vacuolised areas; • Still represented cells with not defined nuclei morphology; • Still represented odontoblasts with homogeneous distribution and stratification of nuclei within the odontoblast layer; • Still present and well recognisable blood vessels and nerves morphology.
7d		Different stages of extracellular matrix degeneration: • Panel A: extracellular matrix with massive disaggregated and vacuolised areas; • Panel B: homogeneous distribution of extracellular matrix without significant signs of vacuolisation; • All three samples: all structures (cells, vessels, and nerves) are visible but not defined nuclei morphology, irregular and wide vascular lumen, less defined morphology of nerve cells; • Still well represented odontoblasts with homogeneous distribution and stratification within the odontoblast layer and cytoplasmic extensions, but irregular morphology of nuclei.

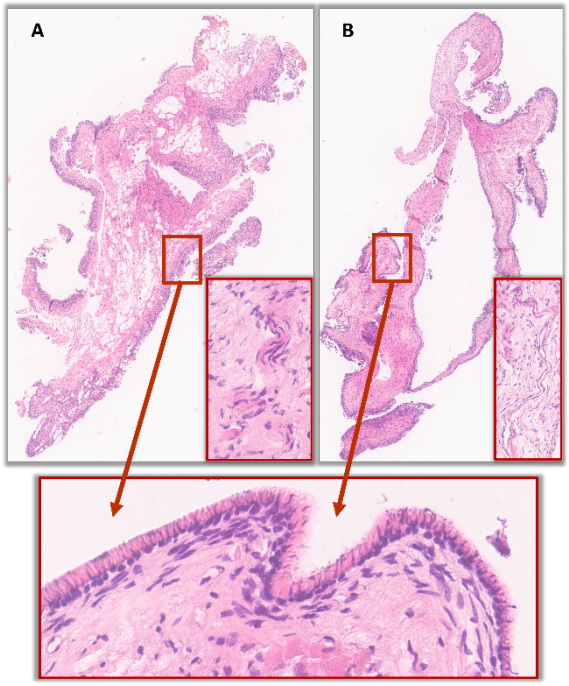
14d		Different stages of extracellular matrix degeneration: • Panel A: extracellular matrix with massive disaggregated and vacuolised areas; • Panel B: homogeneous distribution of extracellular matrix without significant signs of vacuolisation; • All three samples: all structures (cells, vessels, and nerves) are visible but not defined nuclei morphology, irregular and wide vascular lumen, less defined morphology of nerve cells; • Still well represented odontoblasts with homogeneous distribution within the odontoblast layer and cytoplasmic extensions, but thinner stratification and irregular morphology of nuclei.

Figure 1. Manual cell count calculated as the average of the 3 larger ROIs selected for each tooth of the PMI set

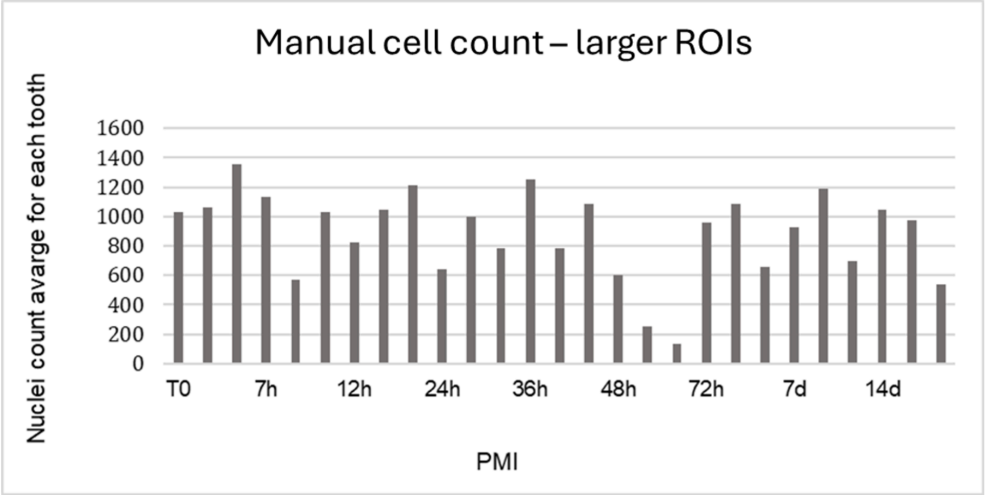


Figure 2. Manual cell density measurement calculated as the average of the 3 teeth for each PMI set, based on larger ROIs

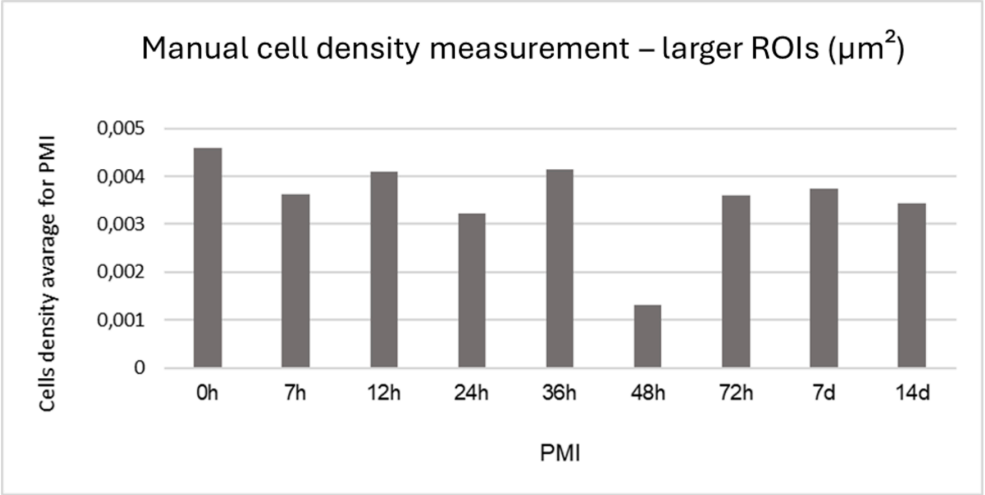


Figure 3. Manual cell count calculated as the average of the 3 smaller ROIs selected for each tooth of the PMI set

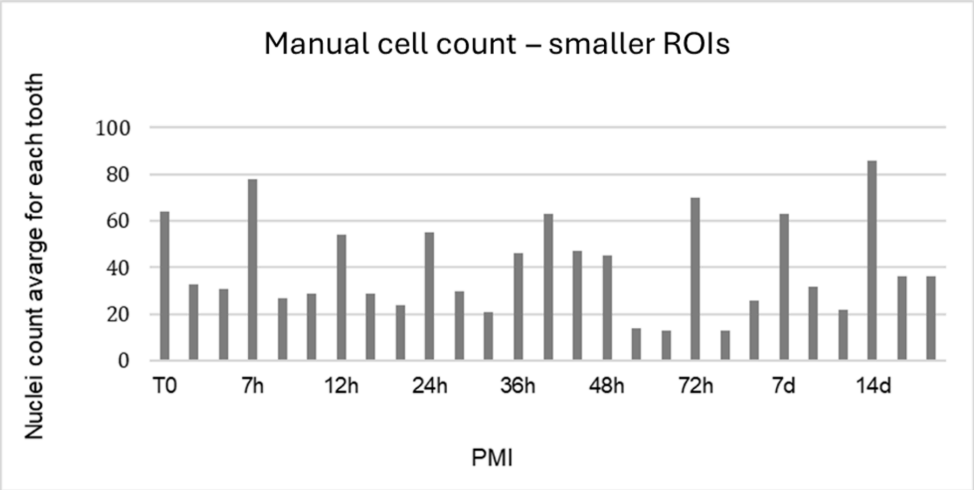


Figure 4. Manual cell density measurement calculated as the average of the 3 teeth for each PMI set, based on smaller ROIs

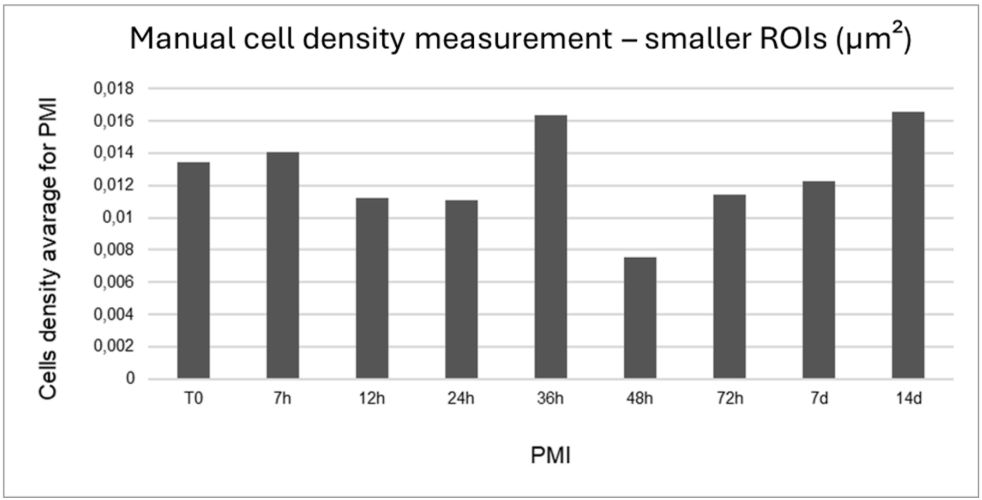


Figure 5. Automatic cell count calculated as the average of the 3 larger ROIs selected for each tooth of the PMI set (in pixels)

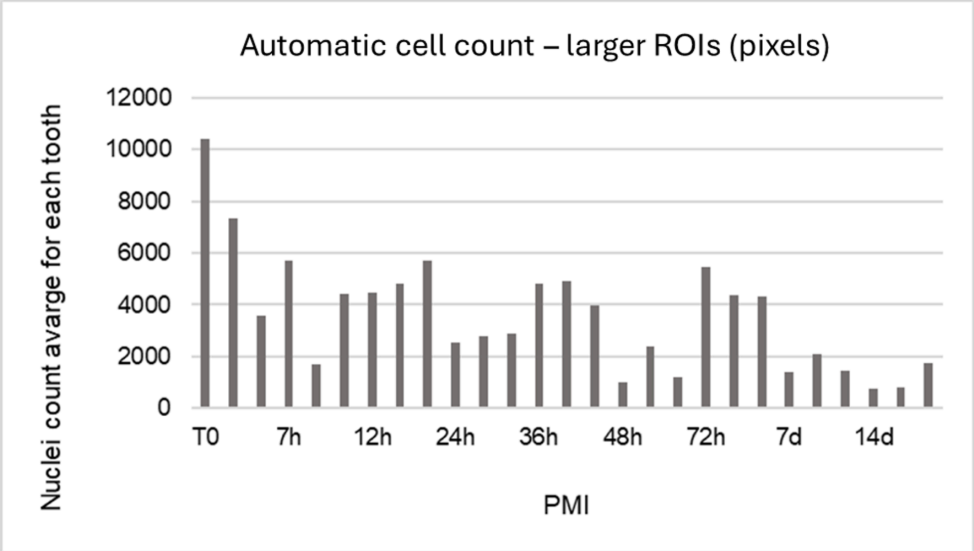


Figure 6. Automatic cell density measurement calculated as the average of the 3 teeth for each PMI set, based on larger ROIs (in pixels)

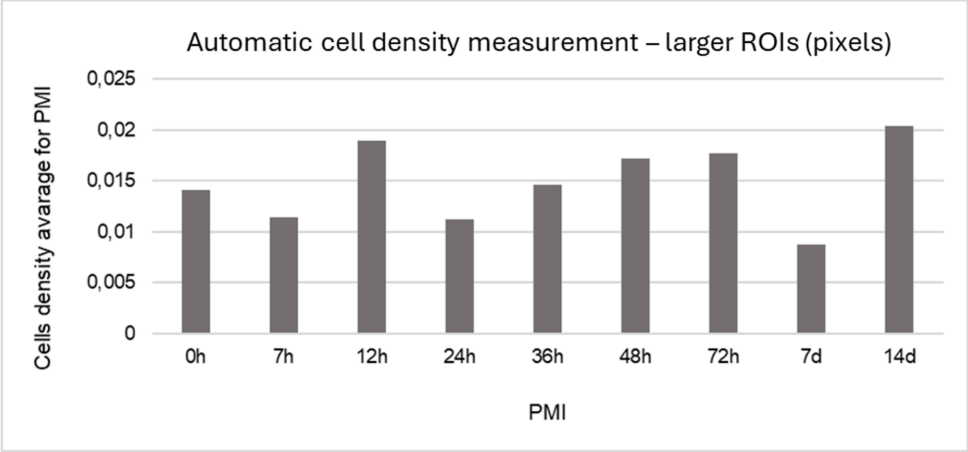


Figure 7. Automatic cell count calculated as the average of the 3 smaller ROIs selected for each tooth of the PMI set (in pixels)

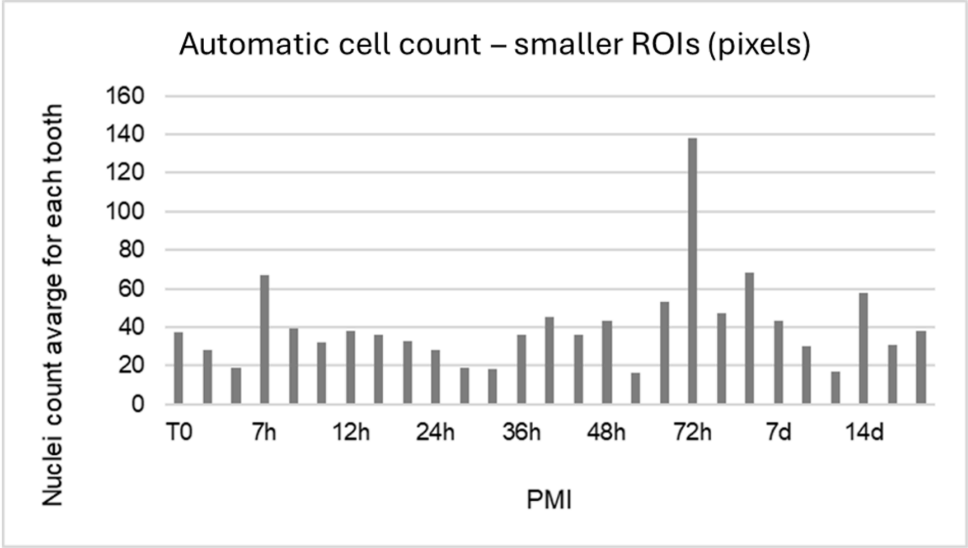
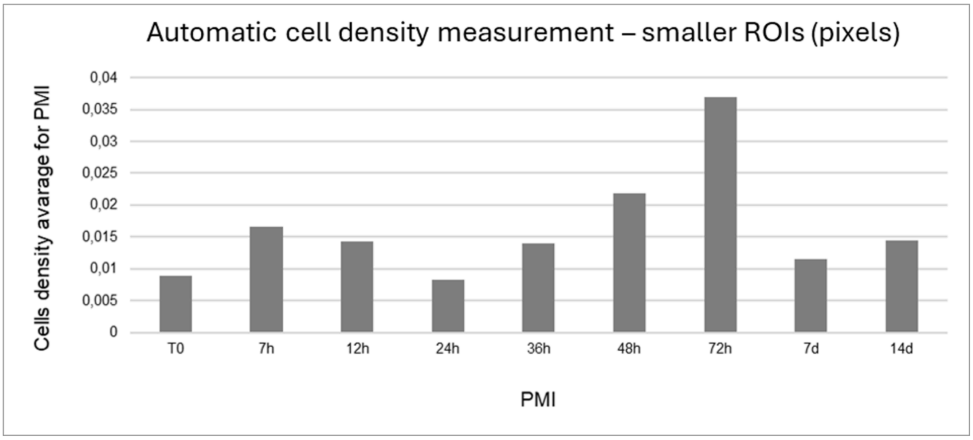


Figure 8. Automatic cell density measurement calculated as the average of the 3 teeth for each PMI set, based on smaller ROIs (in pixels)



DISCUSSION

Estimating the PMI is one of the challenging issues in forensic pathology especially when the corpse is found after the first week following death (late PMI). Teeth can be considered a stable matrix for estimating the time elapsed since death from early to late PMIs since they have great resistance to external damaging factors (such as heat, bacteria, UV light, and moisture) [37-39], and the pulp undergoes morphological, histological and biochemical changes after the interruption of the blood supply [27-36]. Previous studies were promising, but they were not able, either to yield evidence about the timing of the most significant post-mortem (PM) alterations of dental pulp or to indicate definitive hypotheses regarding the occurrence of these alterations [40]. The tissue characteristically consists of four main areas from the outer surface towards the inner: a layer of specialised cells (odontoblast layer) in close relationship with the dentine, an acellular zone formed by fibrils of connective tissue (Weil's basal layer), an area of high cell density composed of differentiated and undifferentiated cells (fibroblasts, macrophages, lymphocytes, inflammatory cells, and young mesenchymal cells) immersed in ECM, and neuro-vascular complex. All these structures seem to demonstrate different stages of degeneration according to the anaerobic metabolism of the various areas of dental pulp [41-42] and the effect of enzymatic activity after death [43-44].

The absence of significant histological changes at T0 (Tab. 2) was expected, and the results confirmed that the dental pulp samples were healthy, and the pulp extraction technique (bur cutting with careful heating control) was conservative and caused no alterations in the pulp tissue.

All samples analysed 7 hours after death (Tab. 2) showed extensive tissue degeneration, characterised by vacuolisation of the ECM but preservation of the other structures (vessels, nerves, nuclear morphology). The results seem to partially confirm the findings of Caballin et al [30] and Bhuyan et al [35] who noted the presence of ECM disaggregation as the main change in the PM pulp within the first 24-72 hrs. Caballin et al [30] attributed this phenomenon to the detachment of the odontoblasts from the dentinal wall. However, a different technique was used to prepare the sample (decalcification of the tooth and microtome cutting) and analysed the whole tooth, consequently, it is not possible to regard their hypothesis as valid. Bhuyan et al [35] reported the initial, localised presence of vacuoles in the ECM due to the release of intra-cellular hydrolytic enzymes and the production of gases by gram-positive anaerobic putrefying bacteria (staphylococci and streptococci) found in the pulp. Since no microbiological analyses was conducted in this study, it is not possible to confirm bacterial involvement and that the ongoing putrefactive process contributed to the late onset (from 24-72 hours PM) of pulp degeneration. However, the obtained results highlight an extensive and early occurrence (within 7 hours of death - Tab. 2) of ECM disaggregation which seems to be in complete contrast with Gawande et al [31] and Carrasco et al [36] who observed a well-preserved nature of pulp tissue with homogeneous distribution of ECM without vacuolization up to 24-72 hours PM.

This significant variability in the rate and extension of the degeneration of the ECM is demonstrated by results obtained for all subsequent PMIs up to 14 days PM, as shown in Table 2, panels A-B-C. It emerged that from 7 hours to two weeks after death it is not possible to correlate the alterations detected in the pulp with the PMI. In fact, samples with almost intact pulp have been observed even for up to 14 days. This finding is consistent with the results obtained by Karthikeyan et al [45] on 25 teeth buried together with fragments of organic tissue (flesh) and examined up to 12 days PM. They reported that most samples analysed at the same PMI showed a similar degree of rate of degradation, but with relevant exceptions that conflicted with the generalisation of histomorphology results for all teeth and seasons [45]. In this study, it is not possible to attribute the variability of results to the putrefactive process of organic tissues around teeth nor to the different characteristics of soil, since the sample teeth were stored outside alveolar bone at room temperature. The study can conclude that the variability observed does not seem to be influenced by the sex of the subject or the type of tooth, being homogeneously distributed between the different PMIs as reported in Tab. 1. Furthermore, not even the temperature variation seemed to influence the rate and extent of the degeneration considering that recorded temperatures showed slight differences as the study was carried out only in the hottest seasons (spring and summer). In fact, the parameter that

seemed to influence the variability of the pulp's response to the PM degenerative phenomena was the age of the subject and of the tooth's physiological age of root maturation, and therefore of the residual dentinogenic activity. In particular, samples 25-26-27 (Tab. 1) belonged to the same 18 year old male subject and were all third molars. Despite being analysed at different PMIs (T0, 12 h, and 36 h, Tab. 2 panels A and C), they never showed signs of degeneration, as did samples 2 and 3 (Tab. 1, Tab. 2 panels B and C) extracted from the same 23 year old male subject at PM 24h. Considering that third molars in males erupt around 17-19 years and complete maturation at 22-23 years of age, these dental elements, although complete, were barely mature with residual dentinogenic activity and not yet subjected to continuous masticatory stress. In fact, samples 2 and 3 showed sound pulp at the same PMI as sample 1, tooth 35 in a 46 year old male subject (Table 1) who on the contrary showed advanced signs of degeneration of the ECM 24 hours after death (Tab. 2, panel A). A similar observation can be made for samples 20-21-22-24 (Tab. 1), all second premolars from a 25 year old female subject. These teeth usually have erupted and completed root calcification around 12-13 in females, therefore they have completed mineralisation at least 10 years before this examination, with no residual primary dentinogenic activity and under masticatory stress and repeated insults. Analysed at three different PMIs (7h, 12h, and 36h, Tab. 1), teeth always showed extensive signs of degeneration (Tab. 2, panels B-C for PMI 12h and A for PMI 36h). Hence in the examined sample the younger the subject, the shorter the time from root completion and the fewer the degenerative alterations of the pulp.

In this sense, a 2015 study [41] on the mechanism of the anaerobic glycolysis in PM bovine dental pulp under oxygen-free conditions applied *in vitro* showed that metabolic exhaustion with added glucose occurred after 80-95 minutes of anaerobic activity, depending on the degree of residual activity of the pulp in the process of dentine deposition. This finding demonstrated that the pulp could cope with the absence of blood supply through the activation of alternative energy mechanisms for a variable time after death according to the maturity stage of the tooth. Therefore, the pulp of subjects of different ages could have different resistance to PM degenerative phenomena, supporting the hypothesis that ageing and the time span since the tooth erupted and completed mineralisation can affect the post-mortem resistance of the pulp and the onset and progression of pulp degeneration after death.

On the contrary, both Bhuyan [35] and Carrasco [36] detected increasing vacuolations of the ECM with time [48h, 72h, 1 month, 3 months, over 6 months], thus suggesting that the variability decreases as the ranges of PMIs considered increase and therefore that histomorphological changes of dental pulp could be a more reliable instrument in late PMIs.

For quantitative measurement of pulp alteration after death, Vavpotič et al [32] found that the estimated time for odontoblast's presence, with a 95% confidence interval, is about 5 days after death with an average drop in the density of 130 odontoblasts/ square millimeter per hour at room temperature. That study is quite different compared to the present research, since Vavpotič et al considered PMIs limited to 5 days after death and the technique required a lengthy preparation of samples with decalcification of tooth, that could have affected results. A faster and simpler technique has been developed here, but both manual and automatic software counting, despite the use of two different units of measurement (μm^2 and pixels), revealed that the density of cells in such a narrow PMI (up to 14 days after death) is not significant (Fig. 2-4-6-8). In the manual measurement of larger ROIs this study obtained a wave-like pattern with density peaks equal to 0.004-0.005 μm^2 for T0, 12h and 36 h PM and a slight constant decrease in density from 72h to 14 days PM (0.003-0.004 μm^2) with a very low peak at 48h after death (0.001-0.002 μm^2). On the contrary, for the measurement of smaller ROIs, the minor peak at 48h is confirmed (0.006-0.008 μm^2) but from 72h to 14 days after death there is even an increase in density (from 0.01-0.012 μm^2 to 0.016-0.018 μm^2) with a recorded density even greater at 14 days PM compared to T0 (0.012-0.014 μm^2). The density measurement with software also showed great variability between the two ROIs, highlighting for larger ROIs a higher density at 14 days PM (0.02 pixels) compared to T0 (0.014 pixels); while for smaller ROIs, a density of 0.09 pixels at T0, with a peak at 72h PM (0.035-0.04 pixels) and a significant decrease at 14 days after death (0.015 pixels) still greater than the value recorded at T0.

These results are partially consistent with those of Carrasco et al [36] who reported the presence of nuclei of all the different cell types of dental pulp up to 6 months, but they revealed a progressive decrease in cell density between 24 hours, 1 month, 3 months and 6 months after death.

The progression of the research is ongoing from the present findings and quite interesting previous reports about the onset and time progression of DNA alteration in dental pulp after death [24]. Caviedes-Bucheli et al [43] demonstrated that in dental pulp, cell activity and DNA expression of lactate-dehydrogenase (LDH) decrease progressively from 89, to 68, up to 41% measured 6, 12, and 24 hours PM, respectively. Boy et al [44] demonstrated that DNA denaturation in dental pulp is continuous for about 72 hours after death and then undergoes minimal progression of degradation by 144 hours, thereby suggesting the usefulness of dental DNA for estimating late rather than early PMIs. This study's results, Tab. 2 - PMI T0, 7 d, and 14 d, demonstrated the presence and activity of odontolblasts after death, suggesting their application in late PMIs (beyond the 2 weeks after death).

Hence late PMIs are under investigation by using easy, quick, and inexpensive histological methods that apply specific colouration for DNA integrity (e.g. Feulgen stain).

The research is limited to two weeks after death to observe the alterations and presence of pulp characteristics in an intermediate PMI. Further studies are already ongoing to extend the PM window of analyses, given the significance of the results obtained.

One of the main limits is the method of extraction and fixation of the pulp, that is an easier and faster method compared to those requiring the tooth decalcification, but can cause an overlap of layers and do not allow a topographic definition of the structures. Moreover, the superposition of nuclei images does not allow repeatable and reliable nuclei counting either manually or via software. Further research will aim at verifying whether the pulp preparation technique with decalcification and cutting is more reliable for standardising the quantitative analysis method on cell density.

CONCLUSION

A pilot study was carried out to analyse the histomorphological changes of 27 PM dental pulps as a possible tool for estimating the PMI up to 14 days after death (intermediate PMI for which the estimate remains complex).

The study demonstrated that dental pulp degeneration appears 7 hours after death, but qualitative histological changes in PM dental pulp are endowed with high variability so it is not possible to generalise the results for early PMIs. ECM, cells, vessels, and fibres are still present 14 days after death even if they show different and uneven degenerative stages. No patterns of degenerative alterations of cells, fibres or nuclei emerged that correlated to different PMIs.

The ageing of the subject and the time span since the tooth erupted and completed mineralisation can affect the post-mortem resistance of the pulp and the onset and progression of pulp degeneration after death.

The quantitative measurements of cell density were not reliable because the cell counts were not standardisable, neither manually nor automatically, due to overlapping layers and nuclei fragmentation. Nevertheless, the presence and activity of odontoblast cells up to 2 weeks after death suggested their application to the study of late PMIs.

Future research should focus on a wider sample analysed at late PMIs (more than 2 weeks after death) and both with pulp extraction-fixation technique and tooth decalcification-cutting method to compare results.

ACKNOWLEDGMENTS

Ana Filomena Aparício da Cruz Maio, MSc, Laboratory of Histomorphology, Faculty of Dental Medicine, University of Lisbon, Portugal; Francisco Salvado, PhD, Faculty of Medicine, University of Lisbon, Portugal; FCT– Fundação para a Ciência e a Tecnologia under the Project UIDB /00006/2020. https://doi.org/10.54499/UIDB/00006/2020; Research Group in Forensic Dental Sciences FORENSEMED from the Research Unit UICOB (Biomedical and Oral Sciences Research Unit), Faculty of Dental Medicine, University of Lisbon, Portugal.

REFERENCES

1. Shrestha R, Kanchan T, Krishan K. *Methods of Estimation of Time Since Death.* [Updated 2023 May 30]. In: *StatPearls [Internet]*. Treasure Island (FL): StatPearls Publishing. 2024; Available from: https://www.ncbi.nlm.nih.gov/books/NBK549867/.
2. Franceschetti L, Amadasi A, Bugelli V, Bolsi G, Tsokos M. *Estimation of Late Postmortem Interval: Where Do We Stand? A Literature Review. Biology.* 2023;12(6):783. https://doi.org/10.3390/biology12060783.
3. Pittner S, Bugelli V, Weitgasser K, Zissler A, Sanit S, Lutz L, Monticelli F, Campobasso CP, Steinbacher P, Amendt J. *A field study to evaluate PMI estimation methods for advanced decomposition stages. Int J Legal Med.* 2020;134(4):1361-1373. doi: 10.1007/s00414-020-02278-0.
4. Pittner S, Bugelli V, Benbow ME, Ehrenfellner B, Zissler A, Campobasso CP, Oostra RJ, Aalders MCG, Zehner R, Lutz L, Monticelli FC, Staufer C, Helm K, Pinchi V, Receveur JP, Geißenberger J, Steinbacher P, Amendt J. *The applicability of forensic time since death estimation methods for buried bodies in advanced decomposition stages. PLoS One.* 2020;15(12):e0243395. doi: 10.1371/journal.pone.0243395.
5. Sironi E, Taroni F, Baldinotti C, Nardi C, Norelli GA, Gallidabino M, Pinchi V. *Age estimation by assessment of pulp chamber volume: a Bayesian network for the evaluation of dental evidence. Int J Legal Med.* 2018;132(4):1125-1138. doi: 10.1007/s00414-017-1733-0.
6. Bartolini V, Pinchi V, Gualco B, Vanin S, Chiaracane G, D'Elia G, Norelli GA, Focardi M. *The iliac crest in forensic age estimation: evaluation of three methods in pelvis X-rays. Int J Legal Med.* 2018;132(1):279-288. doi: 10.1007/s00414-017-1629-z.
7. Pires AC, Vargas de Sousa Santos RF, Pereira CP. *Dental age assessment by the pulp/tooth area proportion in cone beam computed tomography: is medico-legal application for age estimation reliable? J Forensic Odontostomatol.* 2021;39(2):2-14.
8. Oliva G, Pinchi V, Bianchi I, Focardi M, Paganelli C, Zotti R, Dalessandri D. *Three-Dimensional Dental Analysis for Sex Estimation in the Italian Population: A Pilot Study Based on a Geometric Morphometric and Artificial Neural Network Approach. Healthcare (Basel).* 2021;10(1):9. doi: 10.3390/healthcare10010009.
9. Bianchi I, Oliva G, Vitale G, Bellugi B, Bertana G, Focardi M, Grassi S, Dalessandri D, Pinchi V. *A Semi-Automatic Method on a Small Italian Sample for Estimating Sex Based on the Shape of the Crown of the Maxillary Posterior Teeth. Healthcare (Basel).* 2023;11(6):845. doi: 10.3390/healthcare11060845.
10. Wenzlow N, Mills D, Byrd J, Warren M, Long MT. *Review of the current and potential use of biological and molecular methods for the estimation of the postmortem interval in animals and humans. Journal of Veterinary Diagnostic Investigation.* 2023;35(2):97-108. doi:10.1177/10406387231153930.
11. Hachem M, John A, Said HB, Salim A, Zakkir S, Alkash AS, Jimmy D. *Emerging Molecular Approaches for Estimation of Post- Mortem Interval in Medico-Legal Practice. J Mol Genet Med.* 2020;14(5):465. doi: 10.37421/jmgm.2020.14.465
12. Huang W, Zhao S, Liu H, Pan M, Dong H. *The Role of Protein Degradation in Estimation Postmortem Interval and Confirmation of Cause of Death in Forensic Pathology: A Literature Review. International Journal of Molecular Sciences.* 2024; 25(3):1659. https://doi.org/10.3390/ijms25031659.
13. Salerno M, Cocimano G, Roccuzzo S, Russo I, Piombino-Mascali D, Márquez-Grant N, Zammit C, Esposito M, Sessa F. *New Trends in Immunohistochemical Methods to Estimate the Time since Death: A Review. Diagnostics (Basel).* 2022;12(9):2114. doi: 10.3390/diagnostics12092114.
14. Bonicelli A, Mickleburgh HL, Chighine A, Locci E, Wescott DJ, Procopio N. *The 'ForensOMICS' approach for postmortem interval estimation from human bone by integrating metabolomics, lipidomics, and proteomics. eLife.* 2022;11:e83658. https://doi.org/10.7554/eLife.83658.
15. Pasaribu RS, Auerkari EI, Suhartono AW, Auerkari P. *A small RNA, microRNA as a potential biomolecular marker to estimate post mortem interval in forensic science: a systematic review. Int J Legal Med.* 2023;137(5):1313-1325. doi:10.1007/s00414-023-03015-z.
16. Guardado-Estrada M, Cárdenas-Monroy CA, Martínez-Rivera V, Cortez F, Pedraza-Lara C, Millan-Catalan O, Pérez-Plasencia C. *A miRNome analysis at the early postmortem interval. PeerJ.* 2023;11:e15409. doi: 10.7717/peerj.15409.
17. Marrone A, La Russa D, Barberio L, Murfuni MS, Gaspari M, Pellegrino D. *Forensic Proteomics for the Discovery of New post mortem Interval Biomarkers: A Preliminary Study. Int J Mol Sci.* 2023;24(19):14627. doi:10.3390/ijms241914627.
18. Focardi M, Lanzilao L, Bonari A, Lazzeretti M, Lorubbio M, Ognibene A, Gualco B, Fanelli A, Pinchi V. *Improvement in estimation of time since death by albumin and potassium concentrations in vitreous humor. Forensic Sci Int.* 2020;314:110393. doi:10.1016/j.forsciint.2020.110393.
19. Sakr MF, El-Khalek AMA, Mohammad NS, Abouhashem NS, Gaballah MH, Ragab HM. *Estimation of postmortem interval using histological and oxidative biomarkers in human bone marrow. Forensic Sci Med Pathol.* 2023. doi:10.1007/s12024-023-00753-9.
20. Yadav AB, Angadi PV, Kale AD, Yadav SK. *Histological assessment of cellular changes in postmortem gingival specimens for estimation of time since death. J Forensic Odontostomatol.* 2015;33(1):19-26.
21. Pasaribu RS, Auerkari EI, Suhartono AW. *Histogical changes in oral mucosa (gingiva) as a method for estimating post-mortem interval: A literature review. Saudi Dent J.* 2024;36(7):980-983. https://doi.org/10.1016/j.sdentj.2024.05.002
22. Focardi M, Bugelli V, Venturini M, Bianchi I, Defraia B, Pinchi V, Bacci S. *Increased expression of iNOS by Langerhans cells in hanging marks. Australian Journal of Forensic Sciences.* 2020;54(2):177–186. https://doi.org/10.1080/00450618.2020.1817556.
23. Lanzilao L, Bianchi I, Grassi S, Defraia B, Brogi M, Da Ros M, Biagioli T, Fanelli A, Pinchi V, Focardi M. *Biomarkers of traumatic brain injury in vitreous humor: A pilot study. Forensic Sci Int.* 2023;350:111782. doi:10.1016/j.forsciint.2023.111782.
24. Bianchi I, Grassi S, Castiglione F, Bartoli C, De Saint Pierre B, Focardi M, Oliva A, Pinchi V. *Dental DNA as an Indicator of Post-Mortem Interval (PMI): A Pilot Research. Int J Mol Sci.* 2022;23(21):12896. doi:10.3390/ijms232112896.

25. Menon LML, Prado KFB, da Silva RHA. *Histological evaluation of dentine and cementum after different periods of burial: an in vitro study. RSBO (Online)*. 2011;8(2):131-137.
26. Selvan CT, Malkovskiy AV, Vijayaraghavan R, Babu GR, Senthilkumar S. *New insights into odontological exploration of drowning using rat model - A pilot study. J Forensic Odontostomatol*. 2019;37(1):51-62.
27. Ugrappa S, Ajay J. *An Emergence of Dental Tissues in the Forensic Medicine for the Postmortem Interval Estimation: A Scoping Review. Journal of Forensic Science and Medicine*. 2021;7(2):54-60. DOI: 10.4103/jfsm.jfsm_20_20.
28. Ugrappa S, Ajay J. An Overview of the Post-Mortem Interval and its Estimation from Dental Tissues. Annals of the Romanian Society for Cell Biology, 2021;25(4):14903–14910.
29. Yamamoto K. *Experimental study on changes in extracted human teeth in relation to time elapsed. Shikwa Gakuho*. 1959;58:1–21.
30. Caballín A, Peréa B, de Agustín D, Sánchez JA. *Valoración de los cambios histológicos pulpares para la determinación de la data de la muerte. Cient Dent*. 2010;7;1:9-13.
31. Gawande M, Chaudhary M, Das A. *Estimation of the time of death of an individual: -By Evaluating Histological Changes in the Pulp.* LAP LAMBERT Academic Publishing; Illustrated edition. 2013.
32. Vavpotic M, Turk T, Martincic DS, Balazic J. *Characteristics of the number of odontoblasts in human dental pulp post-mortem. Forensic Sci Int*. 2009 Dec 15;193(1-3):122-6. doi: 10.1016/j.forsciint.2009.09.023.
33. Mehendiratta M, Jain K, Boaz K, Bansal M, Manaktala N. *Estimation of time elapsed since the death from identification of morphological and histological time-related changes in dental pulp: An observational study from porcine teeth. J Forensic Dent Sci*. 2015;7:95-100. doi:10.4103/0975-1475.154594.
34. Duffy JB, Skinner MF, Waterfield JD. *Rates of putrefaction of dental pulp in the Northwest coast environment. J Forensic Sci.* 1991 Sep;36(5):1492-502.
35. Bhuyan L, Behura SS, Dash KC, Mishra P, Mahapatra N, *Panda A. Characterization of histomorphological and microbiological changes in tooth pulp to assess post-mortem interval: an observational study. Egypt J Forensic Sci.* 2020;10:19. https://doi.org/10.1186/s41935-020-00193-4.
36. Carrasco PA, Brizuela CI, Rodriguez IA, Muñoz S, Godoy ME, Inostroza C. *Histological transformations of the dental pulp as possible indicator of post mortem interval: a pilot study. Forensic Sci Int*. 2017;279:251-257. doi: 10.1016/j.forsciint.2017.09.001.
37. Mejía C, Herrera A, Sánchez AI, Moreno S, Moreno F. *Behavior in vitro of the dentin-enamel junction in human premolars submitted to high temperatures: prediction of the maximum temperature based on logistic regression analysis. J Forensic Odontostomatol*. 2016;34(1):10-8.
38. Bianchi I, Focardi M, Bugelli V, Gualco B, Pradella F, Pinchi V. *The tongue protrusion in post-mortem fire. J Forensic Odontostomatol*. 2019;37(1):26-31.
39. Sam N, Trivandrum Thanappan S, Joseph AP, Baby Amma Raghavan Pillai V, Mony V. *Histo-morphologic and gravimetric changes of teeth exposed to high temperatures - An in-vitro study. J Forensic Odontostomatol.* 2022;40(3):52-61.
40. Ramos GF, Eskálath MSF, da Silva JFM, Pimenta RS. *Chronothanatognosis, utilization of dental pulp as a tool to determine the death time: a review. Research, Society and Development.* 2021;10(10):e353101018862. DOI: 10.33448/rsd-v10i10.18862.
41. Fisher AK, Walters VE. *Anaerobic glycolysis in bovine dental pulp. Journal of dental research*. 1968;47(5), 717–719. https://doi.org/10.1177/00220345680470050901.
42. Yu CY, Boyd NM, Cringle SJ, Alder VA, Yu DY. *Oxygen distribution and consumption in rat lower incisor pulp. Archives of oral biology*. 2002;47(7), 529–536. https://doi.org/10.1016/s0003-9969(02)00036-5.
43. Caviedes-Bucheli J, Avendaño N, Gutierrez R, Hernández S, Moreno GC, Romero MC, Muñoz HR. *Quantification of lactate-dehydrogenase and cell viability in postmortem human dental pulp. Journal of endodontics*. 2006;32(3), 183–185. https://doi.org/10.1016/j.joen.2005.10.040.
44. Boy SC, Bernitz H, *Van Heerden WF. Flow cytometric evaluation of postmortem pulp DNA degradation. The American journal of forensic medicine and pathology*. 2003;24(2), 123–127. https://doi.org/10.1097/01.PAF.0000069647.55347.3b.
45. Karthikeyan S, Bishen KA, Joshi JK, Kumar P. *Evaluating Postmortem Interval by Assessing Early Putrefactive Changes in Pulp of Buried Teeth in Central India: A Short Study. Journal of Datta Meghe Institute of Medical Sciences University*. 2023;18(4):p 712-716. DOI: 10.4103/jdmimsu.jdmimsu_230_23.